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Elena S. Polovnikova, Ph.D.

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## COMMENTS

The facsimile confirmation of the Patent Office imprinted hereon will acknowledge receipt of:

Serial No: 09/807,809

Filing Date: July 30, 2001

Applicant: Robert David POSSEE et al.

For: Baculovirus Expression System

Papers Submitted: Resubmission of Second Declaration of Robert David Posse, Ph.D.  
including Exhibits A & B; Copy of stamped postcard acknowledging receipt on  
10/19/2005 of Second Declaration of Robert David Posse, Ph.D.

Docket No.: 46309/257438 (23890)

Date Faxed: December 7, 2005

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## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DEC 07 2005

In re Application of:  
Robert David Possee et al.Serial No. 09/807,809  
(National Phase of PCT/GB00/03114)

Filed: July 30, 2001

For: BACULOVIRUS EXPRESSION  
SYSTEM

Examiner: Marvich, Maria

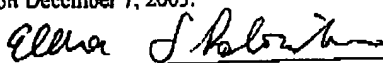
Art Unit: 1636

RESUBMISSION OF SECOND DECLARATION OF ROBERT DAVID POSSEE  
FILED WITH THE SECOND REQUEST FOR CONTINUED EXAMINATION  
AND AMENDMENT AND RESPONSE TO FINAL OFFICE ACTION FILED  
OCTOBER 19, 2005Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

Applicants resubmit herewith copies of the Second Declaration under 37 C.F.R. §1.132 (hereinafter referred to as "the Second Declaration") by Dr. Robert David Possee and Exhibits A and B. The Second Declaration and Exhibits A and B were originally filed on October 19, 2005, with the Second Request for Continued Examination and Amendment and Response to Final Office Action. On December 5, 2005, Examiner Marvich informed the undersigned agent in a voice mail message that the Second Declaration and Exhibits A and B were not on record. Applicants thank the Examiner for the message. Pursuant to a telephone conference with Examiner Marvich on December 7, 2005, applicants resubmit a copy of the Second Declaration and Exhibits A and B.

I hereby certify that this correspondence is being sent to the United States Patent and Trademark Office via facsimile transmission to 571-273-8300 attention Centralized Fax Department on December 7, 2005.



Elena S. Polovnikova - Reg. No. 52,130

**Application Serial No.: 09/807,809**  
**Resubmission of the Second Declaration and Exhibits A and B**  
**Page 2 of 2**

Applicants assert that the Second Declaration and Exhibits A and B were timely filed on October 19, 2005. Attached herewith is a copy of the postcard with the U.S. Patent and Trademark Office official Mailroom Stamp acknowledging receipt on October 21, 2005, of the Second Declaration and Exhibits A and B mailed October 19, 2005. Applicants respectfully request that the Second Declaration and Exhibits A and B be placed on record reflecting that they were timely filed on October 19, 2005.

Applicants respectfully assert that the claims are now in condition for allowance and request that the application be passed to issuance. If the Examiner believes that any informalities that may be corrected by Examiner's amendment remain in the case, or if there are any other issues which can be resolved by a telephone interview, a telephone call to the undersigned agent at (404) 815-6102 is respectfully solicited.

Respectfully submitted,



Elena S. Polovnikova, Ph.D.  
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Reg. No. 52,130

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Attorney Docket: 46309-257438

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The U.S. Patent & Trademark Office official Mailroom stamp  
affixed hereto, acknowledges receipt of the items listed below:

Serial No: 09/807,809

Filing Date: July 30, 2001

Applicant: Robert David POSSEE et al.

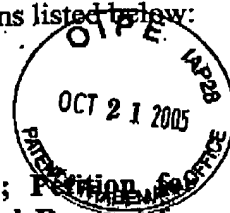
For: Baculovirus Expression System

Papers Submitted: Transmittal of RCE; Petition for  
Extension of Time; Second Amendment and Request for  
Continued Examination; Second Declaration of Robert  
David Posse, Ph.D.; Exhibits A & B; PTO-2038; Figures 5  
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In re Application of:

Robert David Possee et al.

Serial No. 09/807,809

(National Phase of PCT/GB00/03114)

Filed: July 30, 2001

For: BACULOVIRUS EXPRESSION  
SYSTEM

Examiner: Marvich, Maria

Art Unit: 1636

**SECOND DECLARATION OF ROBERT DAVID POSSE, PH.D.  
UNDER 37 C.F.R. §1.132**

I, Robert David Possee, currently residing at 64 Millwood End, Long Hanborough, Witney, Oxon, OX298BY UK, do hereby declare:

1. I am an expert in the field of the invention. I am currently a researcher at NERC CEH Oxford (formerly Institute of Virology and Environmental Microbiology/Institute of Virology). I am a named inventor of U.S. Patent Application 09/807,809 (hereinafter referred to as "the present application"), entitled "Baculovirus Expression System." I am familiar with the application and the Final Office Action mailed by the United States Patent and Trademark Office on April 19, 2005 (hereinafter referred to as "the Final Office Action"). I earned a B.Sc. degree in Biological Sciences with Honours in 1978 at the University of Birmingham, U.K. I earned a Ph.D. degree in virology in 1981 at the University of Warwick, U.K. My *curriculum vitae* is on record in the present application. I have published over one hundred papers and books in the field of biology. The list of the publications are on record in the present application. I am a co-author, with Linda King, who is a named inventor of the U.S. Patent Application Serial No. 09/807,809, of a text book on baculovirus expression systems entitled "*The Baculovirus Expression System; a Laboratory Guide*" (1992), first ed., Chapman and Hall Publishers, London, UK (hereinafter referred to as "my text book"), which was submitted

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18-OCT-2005 10:22 FROM: WITHERS &amp; ROGERS.

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Application Serial No. 09/807,889  
Second Declaration under 37 C.F.R. §1.132  
Page 2 of 5

to the U.S. Patent and Trademark Office with the Information Disclosure Statement filed April 18, 2003.

2. I declare that, prior to the present application, it was not known or obvious that replication-deficient baculovirus vectors could be maintained in bacteria, and then undergo subsequent recombination in the insect cells with the homologous baculovirus sequences.

3. I declare that the method of the present invention, as compared to the method disclosed in *Clark et al.* (hereinafter "*Clark*") results in different properties of the recombinant virus. Specifically, it results in lower contamination with the parental baculovirus.

Enclosed herewith is Exhibit A, which includes Figures 1 and 2 and their legends. Figures 1 and 2 show that when the method of the present invention is used to make recombinant baculoviruses, the recombinant viruses are produced with observable 100% efficiency and without any observable contamination by the parental virus. In particular, Figures 1 and 2 show that co-transfection of all forty four wells of Sf9 cells with the baculovirus vectors of the present invention and transfer vectors containing a gene to be cloned resulted in production of a recombinant protein.

Figure 5 in *Clark* shows an example of producing recombinant baculovirus with a ninety six-well plate system. Sf9 cells were co-transfected with the parent virus DNA and DNA containing a foreign gene. *Clark* states that the resulting recombinant virus was passaged three times on Sf9 cells to amplify and normalize viral titers. When cells from the final passage were stained with Magenta-gluc to detect the presence of recombinant baculovirus, only 78 out of 88 experimental wells (or 89% of the wells) contained the recombinant virus. See *Clark*, brief description of Figure 5, starting in column 6, line 59. This shows that the *Clark* system was observed to be less than 100% efficient, and that, most likely, the parental virus contaminated the recombinant virus stocks.

In contrast, when the method of the present invention is used to make recombinant viruses, the recombinant viruses are produced with observable 100% efficiency and without any observable contamination by the parental virus.

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4. I declare that a vector must comprise a yeast or bacterial origin of replication and an appropriate selectable marker to be maintained in an intermediate host. Clark fails to teach or suggest elements necessary for replication and/or transformation in yeast or bacteria. Applicants conducted experiments in which vectors substantially similar to the Clark vectors were introduced into yeast or bacterial cells by LiCl-mediated transformation or electroporation, respectively. Yeast or bacterial cells failed to support replication of the baculovirus DNA when a yeast-specific or a bacteria-specific replicon was not present in the virus genome.

5. I declare the viral vector in Clark is only suitable for use in the apoptosis deficient host cell line (*T. ni*); the use of such cell line creates a heterogeneous population and reduces production of the desired protein; the vector in Clark cannot be grown in any intermediate host other than the apoptosis deficient insect cell line. If the Clark or similar vectors are introduced into the cells other than an apoptosis-deficient cell line, such as *T. ni*, then virus yields are reduced from about  $10^7$  plaque forming units (pfu) per ml to about  $10^4$  pfu/ml, as shown independently by Lerch and Friesen (1993). See Table 1 in Lerch and Friesen (1993), Exhibit B provided herewith. This has the following consequences for viral DNA production. If the Clark vectors are amplified in *T. ni* cells, then virus production will be nearly the same as for unmodified baculovirus in Sf9 cells. However, if the Clark vectors are amplified in Sf9 cells, which are susceptible to apoptosis, virus yields will be reduced 1000-fold. When purifying viral DNA, it is extracted from the virus particles released from insect cells. Normally, about 100 µg of viral DNA from 500 ml of virus-infected Sf9 cells is expected. With a 1000-fold reduction in the starting material, the Clark system, will yield approximately 0.1 µg of viral DNA from 500 ml of cell culture. This amount of viral DNA is sufficient for transfection of only two cultures of insect cells in order to make recombinant viruses. Twenty four litres (24 L) of virus-infected cell culture will be needed in order to produce 96 viruses in the high-throughput 96-well plate system, as shown in Figure 5 in Clark. Due to the excessive time and labour that will be involved, such large amounts of insect cells culture will be impractical to use for purification of the virus DNA. Thus, only apoptosis deficient cells, such as *T. ni* cells, are, in fact, suitable for parental virus production of Clark vectors.

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Page 4 of 5

6. I declare that *Clark* does not teach, suggest, or provide motivation to use a mutation in ORF1629 to render its vector replication-deficient. Modifications in ORF1629 in *Clark* are additional restriction sites, but they do not render the vector replication-deficient (see column 13, lines 24-26). *Clark* employs the technique of *Kitts and Possee* (1993), which uses linearization by a restriction enzyme of a parental virus. In *Clark*, a full-length copy of ORF1629 is present in the replication-capable parental virus genome prior to linearization, and the deletion in ORF1629 is achieved by enzymatic digestion with a restriction enzyme. The restriction within ORF1629 renders the virus both linearized and replication-deficient. See, for example, Figure 2 in *Clark*. But this deletion is not capable of preventing the parental contamination.

7. I declare that, since *Clark's* baculoviruses are not replication-deficient in insect cells until they are linearized, one of ordinary skill in the art in the field of the present invention would not be motivated to replicate them in cells other than insect cells. *Clark* characterizes the strategies involving heterologous hosts as cumbersome and complicated (see column 2, lines 43-44 and 66-67). *Clark* uses insect cells as the only example of a suitable host in column 6, line 9. The paragraph in column 11, line 50-66, only gives the general definition of the term "host" and does not specifically refer to the *Clark* system.

8. One of ordinary skill in the art in the field of the present invention would not be motivated to use a heterologous host *Clark* system because there is no advantage in doing so in the *Clark* system. *Clark* constructs comprise a p35 mutation and additional restriction sites in ORF1629, which do not prevent replication of the parental virus in the insect cells unless the vectors are digested with a restriction enzyme prior to transfection. If, given a benefit of a hindsight, *Clark* was motivated to use a heterologous host, there would have been no need to in additional step of digesting the vectors prior to transfection into the insect cells.

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Page 5 of 5

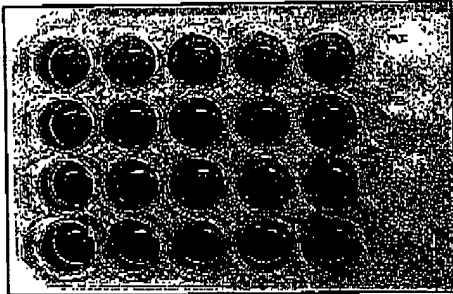
9. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any patent issuing on this application.

RD Possee  
Robert David Possee, Ph.D.

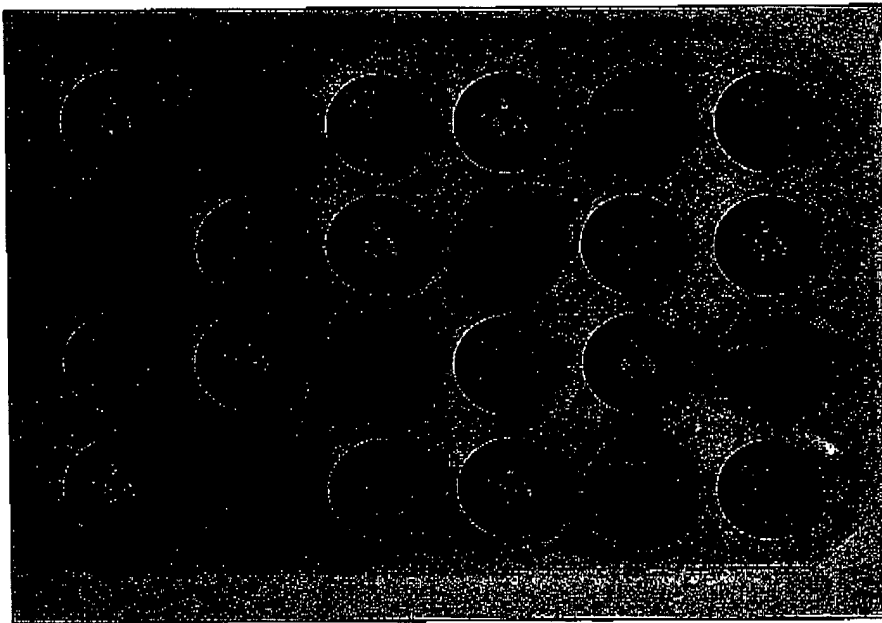
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# Exhibit A



**Figure 1.** Sf9 cells in a 24-well plate were co-transfected with virus DNA from our invention and a transfer vector containing a beta-galactosidase coding region (rows 1-4, wells 1-5). The blue colouration indicates production of the recombinant protein. The four colourless wells on the right side of the plate are negative controls.



**Figure 2.** Sf9 cells in a 24-well plate were co-transfected with virus DNA from our invention and a transfer vector containing a beta-galactosidase coding region, dsred or EGFP. The production of recombinant viruses in each well is demonstrated by the green (EGFP), blue (beta-gal) or red (dsred) colours.

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# Exhibit B

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Nucleic Acids Research, 1993, Vol. 21, No. 8 1753-1760

## The 35-kilodalton protein gene (*p35*) of *Autographa californica* nuclear polyhedrosis virus and the neomycin resistance gene provide dominant selection of recombinant baculoviruses

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Received February 3, 1993; Revised and Accepted March 11, 1993

### ABSTRACT

*Autographa californica* nuclear polyhedrosis virus (AcMNPV) recombinants were constructed to test the effectiveness of the AcMNPV 35-kilodalton protein gene (*p35*) and the bacterial neomycin resistance gene (*neo*) as dominant selectable markers for baculoviruses. Insertion of the AcMNPV apoptosis suppressor gene (*p35*) into the genome of *p35*-deletion mutants inhibited premature host cell death and increased virus yields up to 1200-fold at low multiplicities in *Spodoptera frugiperda* (SF21) cell cultures. When placed under control of an early virus promoter, the bacterial neomycin resistance gene (*neo*) restored multiplication of AcMNPV in the same cells treated with concentrations of the antibiotic G418 that inhibited wild-type virus growth greater than 1000-fold. The selectivity of these dominant markers was compared by serial passage of recombinant virus mixtures. After four passages, the proportion of *p35*-containing virus increased as much as 2,000,000-fold relative to deletion mutants, whereas the proportion of *neo*-containing viruses increased 500-fold relative to wild-type virus under G418 selection. The strength and utility of *p35* as a selectable marker was further demonstrated by the construction of AcMNPV expression vectors using polyhedrin-based transfer plasmids that contain *p35*. Recombinant viruses with foreign gene insertions at the polyhedrin locus accounted for 15 to 30% of the transfection progeny. The proportion of desired viruses was increased to greater than 90% by linearizing the parental virus DNA at the intended site of recombination prior to transfection. These results indicate that *p35* and *neo* facilitate the selection of baculovirus recombinants and that *p35*, in particular, is an effective marker for the generation of AcMNPV expression vectors.

### INTRODUCTION

*Autographa californica* nuclear polyhedrosis virus (AcMNPV) and other members of the family *Baculoviridae* have attracted considerable attention due to their roles as eukaryotic expression vectors and engineered biological insecticides (for recent reviews, see references 1-5). AcMNPV possesses a complex genome consisting of a circular, double-stranded DNA (~131-kb) that encodes 70 to 100 genes. Only a limited number of these genes have been characterized with respect to their role in virus multiplication, high level protein production, and pathogenesis in insects. An important approach for molecular studies of AcMNPV gene function has been the generation of recombinant viruses that contain mutations or gene replacements. New or altered genes are introduced into the virus genome by allelic replacement, a process by which viral DNA sequences are replaced with transfected plasmid sequences through homologous recombination. The same approach is used to construct recombinant virus vectors that carry a foreign gene inserted under control of a strong virus promoter for high level protein expression (3, 4). Since mutant or recombinant viruses are recovered amid a high background of parental virus, a limitation to allelic replacement has been the lack of dominant genes that provide a selectable growth advantage to the desired virus. Such genes would facilitate the amplification and identification of AcMNPV recombinants and are also essential for the isolation of rare virus mutants. Towards the goal of identifying such dominant selectable markers, we have examined two functionally unrelated genes, the AcMNPV 35-kDa protein gene (*p35*) and the bacterial neomycin resistance gene (*neo*), for their effectiveness in the selection and isolation of recombinant baculoviruses.

*p35*, encoded by the EcoRI-S genomic fragment of AcMNPV, is required for wild-type levels of virus multiplication in a cell line-specific manner (6-8). Although the exact function of *p35* is unknown, it directly or indirectly suppresses premature cell death that is the result of apoptosis (6), a host-mediated process

\* To whom correspondence should be addressed

that involves cell lysis and destruction of host DNA. Cultured *S. frugiperda* (SF21) cells are susceptible to virus-induced apoptosis, whereas *Trichoplusia ni* (TN368) cells are not. Production of the budded form of the virus (BV) by *p35*-deletion mutants in SF21 cells is greatly reduced and formation of occluded virus particles is blocked. Subsequent insertion of *p35* at an alternate site within the genome of deletion mutants restores BV production and occlusion-specific gene expression to wild-type levels (8). This finding suggested that *p35* could be used as a dominant selectable marker for recombinant baculoviruses.

In contrast to virus-encoded *p35*, *neo* represents a classical drug resistance marker for dominant selection. This bacterial gene encodes a phosphotransferase that inactivates the protein synthesis inhibitor G418, an aminoglycosidic antibiotic that interferes with eukaryotic 80S ribosome function (9, 10). Insertion of *neo* into the genome of several vertebrate DNA viruses (including vaccinia virus and Epstein-Barr virus) confers dominant selectability in the presence of drug (11–13). In addition, *neo* has been used to develop G418-resistant cell lines derived from invertebrates (14, 15), including the moth *S. frugiperda* which is a permissive AcMNPV host. This suggested that AcMNPV-mediated expression of *neo* would also provide drug resistance and thereby facilitate virus selectivity.

We report here that both *p35* and *neo* (in conjunction with G418) provide a selectable advantage to AcMNPV recombinants. Representing the stronger of the two genetic markers, *p35* readily permitted the selective amplification of recombinant viruses in mixtures that contained a  $10^6$ -fold excess of virus lacking the gene. This demonstrated that it should be possible to select and subsequently identify rare virus mutants containing an inserted copy of this marker. The strength of selectability of *p35* was further utilized for the construction of recombinant viruses by incorporating the marker into allelic transfer plasmids containing a foreign passenger gene. A high proportion of the virus progeny generated after cotransfection with a *p35*-deletion virus consisted of recombinant viruses with the foreign gene inserted at the proper genomic site. Thus, as dominant selectable markers, both *neo* and *p35* have direct application for the isolation of recombinant baculoviruses. As the strongest dominant marker yet identified for AcMNPV, *p35* should be especially useful in facilitating the construction and isolation of virus expression vectors.

## MATERIALS AND METHODS

### Cells and viruses

Established lepidopteran cell lines *S. frugiperda* IPLB-SF21 (SF21) (16) and *T. ni* TN368 (17) were propagated in TC100 growth medium (GIBCO Laboratories) supplemented with fetal bovine serum and 2.6 mg of tryptose broth per mL as described (4). Viruses used included the wild-type L-1 strain of AcMNPV (18), an AcMNPV polyhedrin-deletion mutant (kindly provided by L. K. Miller, University of Georgia), and AcMNPV-deletion mutants  $\Delta 35K$  and  $\Delta 35K/lacZ$  that lack the *p35* promoter and 287 (of 299) codons of the *p35* open reading frame as a result of the deletion of a 950-bp *NruI-SpeI* fragment within the *EcoRI-S* region (8). For infection, cells were inoculated with extracellular BV and rocked for 1 h at room temp, after which the residual inoculum was removed and replaced with growth medium. When indicated, G418 (Geneticin, GIBCO Laboratories) was added to the growth medium.

### Transfer plasmids

To generate *neo*-containing transfer plasmids, *neo* was first placed under control of the promoter for the AcMNPV *p35* gene. To this end, a 1.0-kb *BglIII-SmaI* fragment from plasmid pSV2-*neo* (10) was inserted into the *BamHI* and *SpeI* (end repaired with the Klenow fragment) sites, respectively, of plasmid p35KPRM (19) that contains nucleotides –226 to +12 of the *p35* promoter (RNA start site, +1). Next, the *p35* promoter-*neo* cassette was inserted as a 1.3-kb *XhoI-XbaI* fragment into the corresponding sites of transfer vector pEVocc<sup>+</sup>/PA (5) to generate plasmid pEVocc<sup>+</sup>/*neo*. Transfer plasmid pEV-lacZ/*neo* was constructed by inserting a 3.3-kb *XbaI-KpnI* fragment (end repaired at the *XbaI* site), containing the *Escherichia coli lacZ* gene under control of the polyhedrin promoter, into *XhoI* (end repaired) and *KpnI* sites of pEVocc<sup>+</sup>/*neo*; this replaced the polyhedrin gene with *lacZ*. The *XbaI-KpnI lacZ* fragment was obtained from plasmid pPolylacZ in which the polyhedrin promoter was fused to the *lacZ* gene in the pBluescript (KS) vector (Stratagene). Transfer plasmid pEVocc<sup>+</sup>/35K<sup>+</sup> was constructed by inserting a 1.3-kb *XhoI-XbaI* fragment, containing the complete *p35* gene [including open reading frame and promoter from plasmid p35K-ORF (8)], into the corresponding sites of pEVocc<sup>+</sup>/PA. The transfer plasmid pEV-lacZ/35K<sup>+</sup> was constructed by inserting the 3.3-kb *lacZ*-containing *XbaI-KpnI* fragment (end repaired at the *XbaI* site) into pEVocc<sup>+</sup>/35K<sup>+</sup>, as described for pEV-lacZ/*neo* (see above).

### Generation of AcMNPV recombinants

SF21 cultures ( $2 \times 10^6$  cells) were transfected with viral DNA (1  $\mu$ g) and linearized transfer plasmid (10  $\mu$ g) using Lipofectin (Bethesda Research Laboratories) as previously described (19). When transfections were conducted using whole virus, Lipofectin (30  $\mu$ g) was added directly to a mixture (50  $\mu$ L) of growth medium containing approximately  $6 \times 10^5$  PFU of virus mutant  $\Delta 35K$  and the transfer plasmid. After a 15 min incubation at room temp, the virus-plasmid mixture was added dropwise to SF21 monolayers. Recombinant viruses were harvested 4 days posttransfection, plaque purified using SF21 cells, and characterized by restriction mapping of genomic DNA (data not shown). Linearized viral DNA was used essentially as described previously (20). In brief, 200 ng of  $\Delta 35K/lacZ$  DNA was digested with *Bsu36I* (New England Biolabs), mixed with 2  $\mu$ g of plasmid pEVocc<sup>+</sup>/35K<sup>+</sup> and Lipofectin, then added to  $2 \times 10^6$  SF21 cells. Virus was harvested 2 days posttransfection. Four recombinant viruses (Fig. 1) were generated with the indicated transfer plasmids and parental virus DNAs: 1) *vNeo* was selected by its occlusion-positive (occ<sup>+</sup>) plaque phenotype after transfection with pEVocc<sup>+</sup>/*neo* and an AcMNPV polyhedrin-deletion mutant, 2) *vNeo/lacZ* was selected by its blue, occlusion-negative (occ<sup>-</sup>) phenotype in the presence of Gal (see below) after transfection with pEV-lacZ/*neo* and wild-type AcMNPV, 3)  $\Delta 35K/35K^+$  was selected by its occ<sup>-</sup> phenotype after transfection with pEVocc<sup>+</sup>/35K<sup>+</sup> and  $\Delta 35K/lacZ$ , and 4)  $\Delta 35K/lacZ/35K^+$  was selected by its occ<sup>-</sup> phenotype after transfection with pEV-lacZ/35K<sup>+</sup> and  $\Delta 35K$ . Viruses *vNeo* and *vNeo/lacZ* were identified and isolated in the absence of G418 to avoid *neo* selection.

### Determination of virus titers

When wild-type and *neo*-containing viruses were compared, titers were determined by using SF21 cells in standard plaque assays.

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(4). Due to differences in plaquing efficiency on SF21 cells, titers of both *p35*-deletion mutants and *p35*-containing viruses were determined using TN368 cells; multiplication of AcMNPV recombinants with or without *p35* is indistinguishable in TN368 cells (8). When indicated, expression of  $\beta$ -galactosidase by *lacZ*-containing viruses was visualized by including 100  $\mu$ g of X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) per mL in the agarose overlay. When multiple phenotypes were present on the same plate, plaques were distinguished by using a dissecting microscope.

## RESULTS

*Neo* provides a growth advantage to AcMNPV recombinants in the presence of G418

To test the effectiveness of *neo* as a positive selector for AcMNPV, we constructed two recombinant viruses in which *neo* was inserted at the nonessential polyhedrin locus (Fig. 1B), either adjacent to the polyhedrin gene itself (*vNeo*) or a *lacZ* reporter gene fused to the polyhedrin promoter (*vNeo/lacZ*). In both cases, *neo* was placed under control of the AcMNPV *p35* promoter that has both early and late regulatory elements (19, 21). The effect of G418 on multiplication of *neo*-containing viruses was then compared to that of wild-type virus by inoculating SF21 cultures at a multiplicity of infection (MOI) of 0.05 plaque-forming units (PFU) per cell. This low MOI was used to minimize the frequency with which cells were infected with more than one virus, a requirement for selective amplification of these viruses (see below). The yield of BV accumulated over a 48-h period from cultures treated with G418 was determined by plaque assay. Increasing concentrations of drug resulted in a progressive reduction in the yield of wild-type virus (Fig. 2). At the highest concentrations of G418, wild-type BV yields were reduced more than 1,700-fold. In contrast, BV yields of *neo*-containing viruses,

*vNeo* and *vNeo/lacZ*, were reduced only 2- to 5-fold compared to that in the absence of drug (Fig. 2). This low level of inhibition may be due in part to an observed cytotoxicity of G418 (2.5 mg/mL) in both infected and uninfected cells. Because of its lack of cytotoxicity during infection and the 1,200-fold reduction in wild-type virus yields (Fig. 2), G418 was used at a concentration of 2.0 mg/mL throughout this study. In other experiments, we have noted some variation in the overall level of inhibition of wild-type virus growth which is probably due to differences in commercial G418 preparations (22).

## Differential inhibition of AcMNPV-specific protein synthesis by G418

To examine the mechanism by which G418 interfered with AcMNPV replication, we compared levels of protein synthesis in virus-infected cells treated with G418. Analysis by pulse-labeling with [<sup>35</sup>S]methionine/cysteine indicated that the drug reduced the rate of protein synthesis in cells infected with wild-type virus, but not the *neo*-containing virus *vNeo* (Fig. 3). By 7 h after infection, there was no discernable difference in the rate or pattern of protein synthesis in the presence or absence of G418. However, by 24 h, the synthesis of several prominent virus proteins (including gp64 and polyhedrin) was reduced in cells infected with wild-type virus (lane 6), even though the overall rate of protein synthesis of treated and untreated cultures was comparable. Other virus-specific proteins (see dots) were not affected to the same extent. A similar pattern of selective inhibition by G418 was observed in cultured mammalian cells infected with vaccinia virus (11). By 48 h after infection, G418 dramatically reduced the rate of synthesis of most proteins in wild-type AcMNPV-infected cells (lane 10). In contrast, the drug had no apparent effect on the levels or timing of proteins synthesized in *vNeo*-infected cells. Thus, expression of neomycin-resistance circumvented the inhibition of virus protein synthesis by G418.

## Selective amplification of *neo*-containing viruses

To test whether the resistance to G418 conferred by *neo* was sufficient to selectively amplify AcMNPV recombinants in mixtures where such viruses were in the minority, we monitored

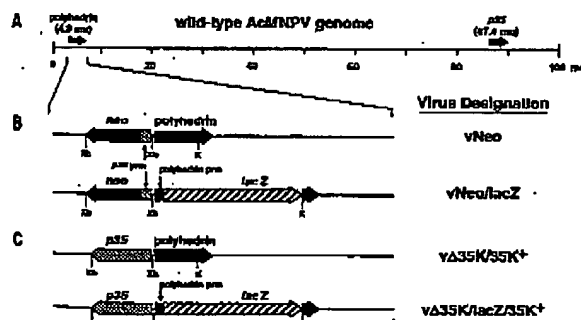


Figure 1. Genome organization of AcMNPV recombinants. (A) Position of the polyhedrin gene (4.3 map units) and *p35* (87.4 map units) on a linear map of the wild-type AcMNPV genome. The gene organization of the polyhedrin locus is illustrated for (B) the *neo*-containing viruses, *vNeo* and *vNeo/lacZ*, and (C) the *p35*-containing viruses, *vΔ35K/35K+* and *vΔ35K/lacZ/35K+*. The *lacZ* gene replaced the polyhedrin gene within viruses *vNeo/lacZ* and *vΔ35K/lacZ/35K+*, while *p35* was removed from its native position within the genome of viruses *vΔ35K/lacZ/35K+* and *vΔ35K/35K+* by deletion of a 950-bp *NruI-SpeI* fragment (87.0–87.7 map units). Shaded arrows depict the promoter (pnm), coding region, and direction of transcription of each gene. The *p35* and polyhedrin genes were controlled by their wild-type promoter. Restriction site abbreviations: K, *KpnI*; M, *XbaI*; Xh, *XhoI*.

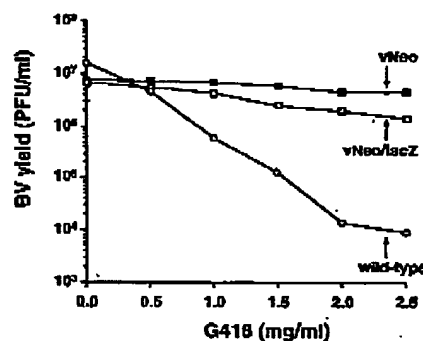


Figure 2. Effects of G418 on production of BV from wild-type and *neo*-containing viruses. SF21 cultures ( $2 \times 10^6$  cells) were inoculated with wild-type, *vNeo*, and *vNeo/lacZ* viruses (0.05 PFU/cell) and covered with growth medium containing the indicated concentration of G418. The medium was collected 48 h after infection and the virus titer was determined by plaque assay using SF21 cells. The yield of progeny BV (PFU/mL) is indicated using a log scale.

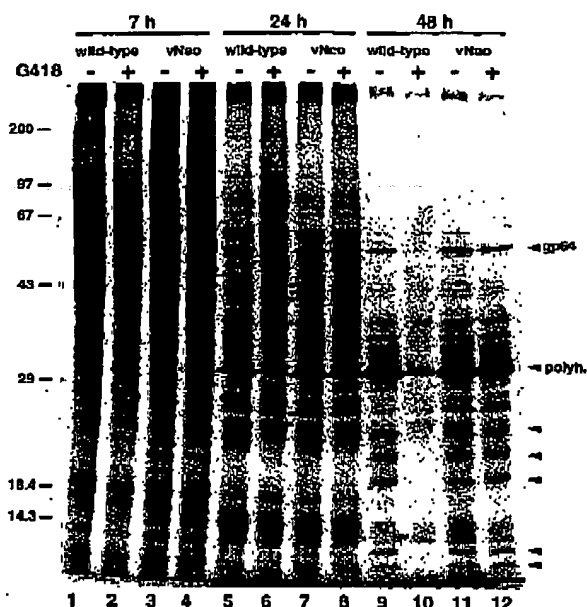


Figure 3. Effects of G418 on protein synthesis in vNeo and wild-type virus infected cells. SF21 cells were inoculated with either wild-type or vNeo viruses (10 PFU/cell) and covered with growth medium with (+) or without (-) G418 (2 mg/mL). At 6, 23, and 47 h after infection, cells were radiolabeled for 1 h with [ $^{35}$ S]methionine-cysteine, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis (23, 24). The positions of molecular weight standards (size in kilodaltons) are shown to the left of the fluorogram. Radiolabeled proteins that were unaffected (dots) or affected (triangles) by G418 are indicated, including polyhedrin (polyh) and the major BV glycoprotein, gp64.

virus multiplication during serial passage of wild-type and *neo*-containing viruses under G418 selection. SF21 cultures were inoculated with experimental mixtures of wild-type virus and vNeo/lacZ using ratios of  $10^3$ ,  $10^4$ , and  $10^5$  to 1, respectively. To minimize helper virus activity, expected to occur when both viruses infect the same cell, the first infection was initiated at a low MOI (0.05 PFU/cell); subsequent passages represented blind (unknown MOI) inoculations using growth medium from the previous infection. The level of each virus was determined by plaque assay in the presence of X-Gal to distinguish vNeo/lacZ (blue, *occ*<sup>-</sup> plaques) from wild-type virus (colorless, *occ*<sup>+</sup> plaques). Over four serial passages, vNeo/lacZ increased progressively (Fig. 4). The final ratio of wild-type virus to vNeo/lacZ for the starting mixtures ( $10^3$ ,  $10^4$ , and  $10^5$  to 1) was 7, 81, and 200 to 1, respectively. Thus, the greatest overall increase in vNeo/lacZ (4,200-fold) occurred within the virus mixture having the lowest starting ratio,  $10^5$  to 1 (Fig. 4C). In general, four serial passages resulted in a 100- to 500-fold increase in the proportion of *neo*-containing virus. In contrast to the *neo*-containing virus, the level of wild-type virus remained steady after a small initial increase in all mixtures. As shown in Fig. 2, G418 reduces the yield of wild-type virus more than 1000-fold, but fails to block virus growth completely. Thus, the nearly constant levels of wild-type virus during serial passage suggested that the yield of wild-type progeny was about the same as that used for inoculum. This was supported by other

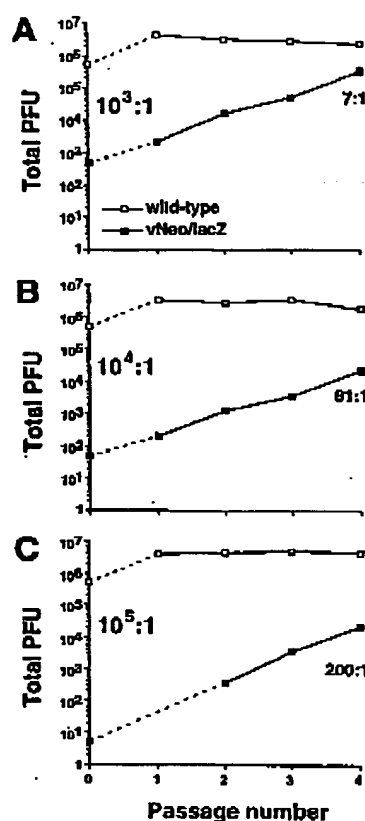


Figure 4. Selective amplification of *neo*-containing recombinant vNeo. Serial passage. SF21 cultures ( $10^7$  cells) were inoculated at a constant MOI (PFU/cell) with the indicated mixtures A)  $10^3$ :1, B)  $10^4$ :1, and C)  $10^5$ :1 wild-type and vNeo/lacZ viruses, respectively, and covered with growth medium containing G418 (2 mg/mL). The medium was collected 30 h after infection. 4 mL was used to inoculate a subsequent culture ( $10^7$  cells). The virus in the remaining medium (1 mL) was determined by plaque assay using SF21 and X-Gal. The yield (Total PFU) of wild-type and vNeo/lacZ viruses for each of the four successive infections using a log scale. The initial level of virus is indicated on the Y axis and the determined ratio of wild-type to vNeo for the last passage is indicated on the right.

Table 1. BV yields of AcAdNPV recombinants with or without p35

Virus	BV production (total PFU)	Fold increase over vA35K
wild-type	$1.2 \pm 0.31 \times 10^7$	1000
vA35K	$1.2 \pm 0.26 \times 10^7$	1
vA35K/35K <sup>+</sup>	$4.4 \pm 0.56 \times 10^6$	370
vA35K/lacZ/35K <sup>+</sup>	$1.4 \pm 0.26 \times 10^7$	1200

<sup>a</sup> SF21 cultures ( $2 \times 10^6$  cells) were infected with the indicated virus (10 PFU per cell). The yield of budded virus (BV) produced after 48 h was determined by plaque assay using TN368 cells. Values shown represent the average of three independent infections.

experiments in which the level of wild-type virus produced after a single, low MOI inoculation (0.05 PFU/cell) of G418 to cells was similar to the input virus (data not shown).

Figure 4 shows the index of the inoculum on the Y axis and the determined ratio of wild-type to vNeo for the last passage is indicated on the right.

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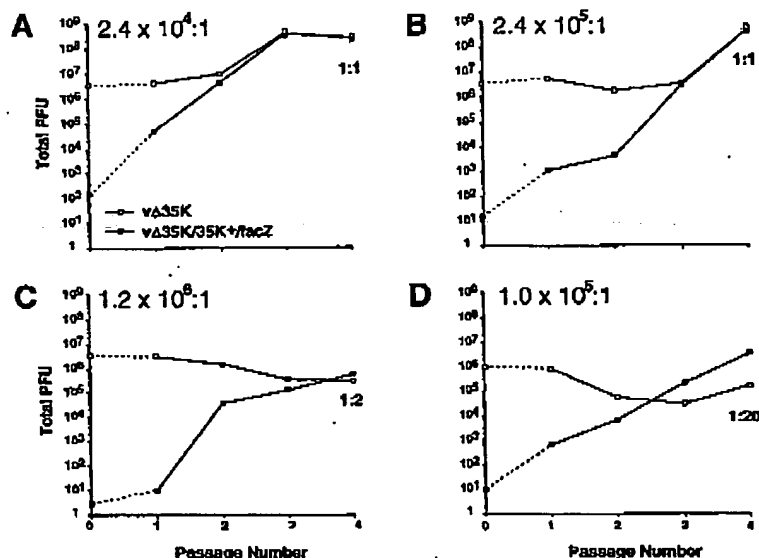


Figure 5. Selective amplification of the  $p35$ -containing virus  $v\Delta35K/lacZ/35K^+$  by serial passage. Replicate SF21 cultures ( $5 \times 10^6$  cells/plate) were inoculated with the indicated mixtures A)  $2.4 \times 10^4:1$ , B)  $2.4 \times 10^5:1$ , C)  $1.2 \times 10^6:1$ , and D)  $10^5:1$  of viruses  $v\Delta35K$  and  $v\Delta35K/lacZ/35K^+$ , respectively. Cells were inoculated at an MOI of 0.7 PFU/cell (A, B, and C) or 0.1 PFU/cell (D) using virus stocks with titers determined by plaque assay using TN368 cells. After a 1-h adsorption, the inoculum was removed and replaced with growth medium (5 mL). All of the medium was collected 48 h after infection and used to inoculate the next culture. The level of progeny viruses was determined in a parallel infection by plaque assays using TN368 cells and X-Gal. The initial level of input viruses is indicated on the Y axis (log scale) and virus yields (Total PFU) are shown for four passages. The determined ratio of  $v\Delta35K$  to  $v\Delta35K/lacZ/35K^+$  for the last passage is indicated on the right.

Table 2. Proportion of viruses generated by transfection with transfer plasmid  $pEV-lacZ/35K^+$  and parental virus  $v\Delta35K$ .

Virus	SF21 plaque phenotype	Fraction of Total Plaques (%) <sup>a</sup>	
		Purified DNA <sup>b</sup>	Intact Virus <sup>c</sup>
$v\Delta35K/lacZ/35K^+$ (desired)	blue, occ <sup>-</sup>	15	30
$v\Delta35K$ (parental)	colorless, occ <sup>-</sup>	41	35
$lacZ^+$ , $35K^+$ (other)	blue, occ <sup>+</sup>	20	10
$35K^+$ (other)	colorless, occ <sup>+</sup>	24	25

<sup>a</sup> Virus was harvested 4 days posttransfection and screened by plaque assay using SF21 cells in the presence of X-Gal; occlusion-negative (occ<sup>-</sup>), occlusion-positive (occ<sup>+</sup>). Values shown represent the average of two independent transfections which did not vary more than  $\pm 3\%$ .

<sup>b</sup> Purified, circular  $v\Delta35K$  DNA (1  $\mu$ g) was transfected with  $pEV-lacZ/35K^+$  (10  $\mu$ g) using  $2 \times 10^6$  SF21 cells.

<sup>c</sup> An equal volume of  $v\Delta35K$  BV ( $6 \times 10^5$  PFU) was substituted for purified viral DNA.

$p35$  confers a growth advantage to AcMNPV recombinants  $p35$ -deletion viruses cause premature cell lysis of SF21 cultures and produce low yields of BV (6, 8). To test the effectiveness of  $p35$  as a selectable marker, we examined the growth properties and selectability of AcMNPV recombinants (Fig. 1C) in which a copy of  $p35$  under control of its own promoter was inserted at the polyhedrin locus of  $p35$ -deletion mutant  $v\Delta35K$ . When  $p35$  was inserted either adjacent to the polyhedrin gene itself ( $v\Delta35K/35K^+$ ) or adjacent to the  $lacZ$  reporter gene fused to

the polyhedrin promoter ( $v\Delta35K/lacZ/35K^+$ ), the accumulated yield of BV over a 48-h period was 370- and 1200-fold higher, respectively, than that of  $v\Delta35K$  (Table 1). Using low MOI (0.05 PFU/cell) inoculations, the accumulated BV yield of  $v\Delta35K$  was 1000-fold lower than that of wild-type virus. Thus, insertion of  $p35$  restored growth of  $p35$ -deletion mutant to levels similar to that of wild-type virus. Other studies have indicated that growth differences between viruses that contain or lack  $p35$  vary with the MOI (8).

## Selective amplification of p35-containing viruses

To assess the selectability conferred by p35, we monitored virus multiplication during serial passage of mixtures of deletion mutant vΔ35K and vΔ35K/lacZ/35K<sup>+</sup>, where the later virus was in the minority. SF21 cultures were inoculated at an MOI of 0.7 PFU/cell and the yield of each virus during serial passage was determined. Plaque assays were conducted using TN368 cells and X-Gal to distinguish vΔ35K (colorless, occ<sup>+</sup> plaques) from vΔ35K/lacZ/35K<sup>+</sup> (blue, occ<sup>-</sup> plaques). For each of the virus mixtures, the level of p35-containing virus, vΔ35K/lacZ/35K<sup>+</sup> increased dramatically (Figs. 5A-5C). After four consecutive passages, the ratio of vΔ35K to vΔ35K/lacZ/35K<sup>+</sup> was 1, 1, and 0.5 to 1 for the starting ratios of  $2.4 \times 10^4$ ,  $2.4 \times 10^5$ , and  $1.2 \times 10^6$  to 1, respectively. This corresponded to 24,000-fold, 240,000-fold, and  $2.4 \times 10^6$ -fold increases in the proportion of p35-containing virus.

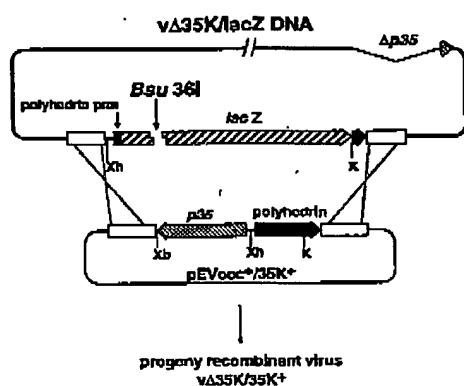


Figure 6. Strategy for gene replacement using linear vΔ35K/lacZ virus DNA. Homologous recombination between vΔ35K/lacZ virus DNA (top) and transfer plasmid pEVocc<sup>+</sup>/35K<sup>+</sup> (bottom) replaced the viral lacZ gene with a functional polyhedrin gene. The resulting recombinant virus (vΔ35K/35K<sup>+</sup>) exhibited an occlusion-positive (occ<sup>+</sup>) plaque phenotype. vΔ35K/lacZ DNA was digested with Bsu36I that linearized the genome at a unique site within the lacZ gene. Shaded arrows depict the promoter (p35), coding region, and direction of transcription of each gene; the p35 and polyhedrin genes were controlled by their wild-type promoter. DNA sequences (open boxes) for homologous recombination between the virus and transfer plasmid are located on either side of the polyhedrin locus. Restriction site abbreviations are listed in the legend to Figure 1.

During later passages when the levels of viruses vΔ35K/lacZ/35K<sup>+</sup> and vΔ35K were high and subsequent MOIs were boosted proportionately, both viruses increased in parallel (Fig. 5A and 5B). This suggested that vΔ35K/lacZ/35K<sup>+</sup> provided helper virus activity for vΔ35K. To reduce such activity, we initiated a serial passage at a lower MOI (0.1 PFU/cell) using an initial vΔ35K to vΔ35K/lacZ/35K<sup>+</sup> ratio of  $10^5$  to 1. After four passages (Fig. 5D), a higher fraction of vΔ35K/lacZ/35K<sup>+</sup> ( $20$  to  $1$ ) was obtained, even though the overall increase ( $2 \times 10^6$ -fold) of this virus was identical to that obtained with a higher MOI (Fig. 5C). Thus, by maintaining a low MOI through consecutive passages, the proportion of p35-containing virus would be expected to increase even further and also minimize the potential for defective virus formation.

## Use of p35 as a selectable marker in the generation of AcMNPV recombinants

The relative strength of selectivity conferred by p35 suggested that it would expedite the recovery of recombinant viruses with new (foreign) DNA inserted into the viral genome by allelic replacement, a method that typically yields desired recombinants at a frequency of 0.1% to 1% of the total progeny virus (4). To test this possibility, we transfected SF21 cells with deletion mutant vΔ35K and a polyhedrin-based transfer plasmid (pEV-lacZ/35K<sup>+</sup>) that contains p35 and its promoter immediately adjacent to the lacZ reporter gene under control of the polyhedrin promoter. The proportion of desired recombinants containing lacZ and p35 inserted at the polyhedrin locus (resulting in loss of the polyhedrin gene) was determined. Since acquisition of p35 restores polyhedrin promoter-mediated gene expression (8), the desired vΔ35K/lacZ/35K<sup>+</sup> recombinants (blue, occ<sup>-</sup> plaques) were distinguished from parental vΔ35K (colorless, occ<sup>+</sup> plaques) by plaque assay using SF21 cells. When purified vΔ35K DNA was used, desired recombinants comprised 15% of the transfection progeny, compared to parental virus that comprised 41% (Table 2). To simplify transfections, viral DNA was replaced with intact vΔ35K BV by using an equal volume of vΔ35K-containing growth medium. When intact virus was used, the proportion of desired recombinants increased to 30% of the total progeny (Table 2).

While more than 60% of the transfection progeny exhibited plaque phenotypes consistent with acquisition of p35, the phenotype of more than half of these recombinant viruses (colorless, occ<sup>+</sup> and blue, occ<sup>+</sup> plaques) indicated that insertion occurred at sites other than the polyhedrin locus (Table 2). Southern blot analysis of viral DNA from representative colorless,

Table 3. Proportion of AcMNPV recombinants using linearized and circular DNA from vΔ35K/lacZ<sup>a</sup>

Virus DNA	Fraction of Total Plaques (%)							
	SF21		Other <sup>c</sup>		TN368		Other <sup>c</sup>	
	Desired <sup>b</sup>		A.	B.	Desired <sup>b</sup>		A.	B.
Circular	97	17	24	83	76	3	3	97
Linearized	96	87	4	13	92	82	8	18

<sup>a</sup> SF21 cultures ( $2 \times 10^6$  cells) were transfected with Bsu36I- or mock-digested (circular) vΔ35K/lacZ DNA (0.2 μg) and pEVocc<sup>+</sup>/35K<sup>+</sup> (2 μg). The growth medium was harvested 2 days posttransfection and scored for recombinant viruses by plaque assay using the indicated cell lines (SF21 or TN368) in the presence of X-Gal. The results of two independent experiments (A. and B.) are shown.

<sup>b</sup> Colorless, occ<sup>+</sup> plaques were scored as the desired recombinant, vΔ35K/35K<sup>+</sup>.

<sup>c</sup> Occ<sup>-</sup> or blue (lacZ-containing) plaques, representing either parental virus vΔ35K/lacZ or undesired recombinant viruses, were scored as other.

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occ<sup>+</sup> plaques confirmed the insertion of *p35* at alternate genomic sites (data not shown). Thus, these undesired recombinants were probably generated through low-level non-homologous recombination between the transfer plasmid and parental *vΔ35K* that were selectively amplified due to acquisition of *p35*. In contrast, upon replating on SF21 cells, 90% of the blue, occ<sup>+</sup> plaques generated multiple plaque phenotypes (data not shown). This suggested that most of the blue, occ<sup>+</sup> plaques represented mixed infections in which polyhedrin and *lacZ* were encoded by separate viruses.

#### Linearization of viral DNA facilitates site-specific insertion of the *p35* selectable marker

To direct recombination to a single site and thus reduce the fraction of recombinants in which the *p35* marker inserted at undesired locations, we determined the effect of linearizing parental virus DNA prior to transfection. It has been shown previously that plasmid recombination across the endonuclease-generated gap of linearized viral DNA produces a higher fractional yield (up to 30%) of recombinant viruses, most likely through the recircularization of DNA that is required for infectivity (20). To this end, DNA from deletion mutant *vΔ35K/lacZ* was linearized with *Bsu36I* at a unique site within the *lacZ* gene, and transfected with transfer plasmid pEVocc<sup>+</sup>/35K<sup>+</sup>. Plasmid recombination across the resulting gap was predicted to generate a virus (*vΔ35K/35K<sup>+</sup>*) in which the *lacZ* gene of parent *vΔ35K/lacZ* was replaced by the polyhedrin and *p35* genes (Fig. 6).

Compared to circular (mock-digested) DNA, linearized virus DNA yielded a higher proportion of desired *vΔ35K/35K<sup>+</sup>* viruses (Table 3). When plated on SF21 cells, up to 96% of the progeny was comprised of desired *p35*-containing recombinants; these viruses produced colorless, occ<sup>+</sup> plaques that were distinguished from the occ<sup>-</sup> plaques of parent *vΔ35K/lacZ*. Restriction mapping of twelve randomly picked viruses with a colorless, occ<sup>+</sup> phenotype demonstrated that each had the predicted genome structure for *vΔ35K/35K<sup>+</sup>* (data not shown). The high proportion of desired recombinants was confirmed by plaque assay using TN368 cells in which the desired (colorless, occ<sup>+</sup>) and parental (blue, occ<sup>-</sup>) viruses have similar plaquing efficiencies. By this analysis, linearized DNA increased the proportion of desired viruses from 3% to about 87% (Table 3). When linearized *vΔ35K/lacZ* DNA was transfected with a transfer plasmid that contained a defective copy of *p35* (pEVocc<sup>+</sup>/35K<sup>Δ35K</sup>) (8), no desired recombinant viruses were detected using TN368 cells (data not shown). In contrast, transfections with circular *vΔ35K/lacZ* DNA and the same transfer plasmid yielded approximately 1% desired recombinants. This finding demonstrated that a functional copy of *p35* was required for the high proportion of desired recombinant viruses, and that linearization of parental DNA alone was not responsible.

#### DISCUSSION

In this report, we have identified two genes, AcMNPV-encoded *p35* and *neo*, that confer dominant selectivity to baculoviruses upon insertion into the viral genome. Use of these positive genetic markers makes it possible to selectively amplify recombinant (or mutant) viruses amid a high background of parental virus. Thus, by facilitating the generation of virus mutants, these genes will be useful for continued studies on baculovirus gene function and

organization. The especially strong selectivity provided by *p35* also facilitates the construction and isolation of AcMNPV expression vectors.

#### *Neo* as a selectable marker for AcMNPV recombinants

When added at the time of inoculation, G418 reduced the accumulated yield of wild-type BV greater than 1,000-fold and thus demonstrated that this antibiotic is an effective inhibitor of AcMNPV multiplication (Fig. 2). Our preliminary results suggested that G418 inhibits virus growth by reducing the synthesis of selected late viral proteins. In contrast, when *neo* was placed under control of the promoter for *p35*, late virus protein synthesis and BV production was restored; BV yields were only 2- to 5-fold lower than that in the absence of drug (Fig. 2). Thus, virus-mediated expression of *neo* was capable of conferring G418 resistance. As demonstrated by serial passage of virus mixtures in the presence of G418, *neo* also conferred dominant selectivity to AcMNPV recombinants. As high as a 500-fold increase in the *neo*-containing recombinant *vNeo/lacZ* (relative to wild-type virus) was obtained over four consecutive passages (Fig. 4). We have not yet tested whether a different AcMNPV promoter would improve virus selectivity. The strong p10 promoter of *Galleria mellonella* nuclear polyhedrosis virus has been used to direct *neo* expression in infected *Lymantria dispar* cells (25). Although recovery of *neo*-containing virus required a prolonged period of G418 selection (10 serial passages of 6 days each), this indicated that *neo* can be used for selection of other baculoviruses. *Neo* selectivity may therefore be useful in studies that monitor the movement of marked transposons between different baculoviruses and their invertebrate hosts (see below).

*Neo* is the first drug resistance marker for positive selection of baculoviruses. Recently, the herpes simplex virus type 1 thymidine kinase gene (HSV1-tk) was shown to be an effective marker for negative selection of baculoviruses (26). Using a strategy similar to that developed for vaccinia virus vectors (22, 27), AcMNPV-mediated expression of HSV1-tk resulted in conferred sensitivity of viral replication to nucleoside analogues, including Ganciclovir (28). Virus replication was restored when drug sensitivity was lost by replacement of tk with a foreign gene, thereby allowing selective amplification of tk<sup>-</sup> recombinants. In contrast to tk that requires prior insertion of the marker into the virus genome, *neo* provides selectivity upon insertion that is not limited at a single locus in the AcMNPV genome.

#### *p35* as a dominant selectable marker for AcMNPV

The high levels of BV production in SF21 cells by *p35*-containing viruses compared to *p35*-deletion mutants suggested that this virus-encoded gene would provide a powerful selection for recombinant viruses. This was first demonstrated by serial passage of virus mixtures in which *p35*-containing viruses were selectively amplified. In the most dramatic case, virus *vΔ35K/lacZ/35K<sup>+</sup>* was amplified more than two million-fold relative to a deletion mutant in only four passages (Fig. 5). These results also demonstrated that *p35*-containing viruses could be amplified and subsequently identified within mixtures that contained more than a million-fold excess of virus lacking the gene. Thus, *p35* should be an effective marker for amplification and isolation of rare virus mutants. For purposes of generating virus mutants, *p35* has a distinct advantage because of its position-independence that permits marker insertion at different sites

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within the AcMNPV genome. Using this approach, we have generated several mutants within the AcMNPV *HindIII*-K/Q region with allelic transfer plasmids that contain *p35* linked to altered virus sequences for replacement within the virus genome (29). The position-independent expression of *p35* may also be useful for studies of lepidopteran transposons that are capable of integrating within the AcMNPV genome (1, 24, 30). By engineering such mobile elements to include *p35*, it is expected that rare transposon insertions into *p35*-deletion mutants can be detected and subsequently characterized. Studies are underway to test this possibility.

#### *p35* as a dominant selectable marker for the generation of AcMNPV expression vectors

Compared to *neo*, *p35* provided dramatically higher levels of selective amplification. We have exploited this selectivity as a means to improve the recovery of recombinant virus vectors for foreign protein expression. When *p35* was incorporated into transfer plasmids containing a foreign gene (*lacZ*) under control of the polyhedrin promoter, approximately 15–30% of progeny obtained after transfection with deletion virus  $\Delta$ 35K consisted of desired recombinant viruses in which the polyhedrin gene was replaced by the foreign gene (Table 2). This represented a 15- to 300-fold increase in the frequency (0.1 to 1%) with which recombinants are recovered using transfected (circular) viral DNA (4) and approximately the same as that obtained when viral DNA is linearized prior to transfection (20). By linearizing the DNA from *p35*-deletion viruses, the proportion of desired recombinants increased even further, ranging from 82 to 96% (Table 3). This represents the highest fractional yield for desired recombinant plaques yet reported in the literature. By using liposome-mediated transfection methods, only small quantities of *p35*-mutant DNA (100–200 ng) are required to obtain yields of  $10^3$  to  $10^5$  recombinant viruses per mL of growth medium when harvested 2 to 4 days after transfection. This conserves parental virus DNA which is produced at levels 10-fold lower than wild-type virus in *S. frugiperda* cells.

Several new methods for baculovirus expression vector construction are available. These include the use of endonuclease-linearized viral DNA (20, 31), the Cre-loxP recombination system (32), genome selection in *Saccharomyces cerevisiae* (33), and HSV1-tk-mediated drug selection (26). Use of allelic transfer plasmids that contain *p35* as a selectable marker provides an alternative and simple approach. The same cell line (*S. frugiperda* SF21) can be used for transfection, purification of recombinant viruses, and production of foreign protein. Moreover, *p35*-mediated selection does not involve potentially mutagenic drugs. Using  $\beta$ -galactosidase as a reporter, we have found that the presence of *p35* immediately adjacent to the polyhedrin promoter has little, if any, effect on the high level of foreign protein expression by AcMNPV vectors (8). In addition, the recovery rate of recombinant viruses is sufficiently high that a pure virus stock can be produced after a single round of plaque isolation (our unpublished results). This has reduced the time required to ascertain the level of foreign protein production to about 2–3 weeks as demonstrated by the construction of *p35*-containing virus vectors by our own and other laboratories. A potentially useful feature of *p35* as a selectable marker is the relatively high rate (~30–50%) with which desired recombinant viruses are recovered when intact BV of the *p35*-deletion mutant  $\Delta$ 35K is used in transfections rather than virus DNA. With

appropriate modifications, it should be possible to eliminate the need for purified virus DNA, thereby simplifying transfections and reducing the expense of expression vector construction.

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